United States
Environmental Protection
Agency

Environmental Monitoring and
Support Laboratory
Cincinnati OH 45268

Research and Development

EPA-600/4-85/076

EPA

TEST METHODS FOR ESCHERICHIA COLI AND ENTEROCOCCI IN WATER BY THE MEMBRANE FILTER PROCEDURE

DISCLAIMER

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Environmental measurements are required to determine the quality of ambient waters and the character of wastewater effluents. The Environmental Monitoring and Support Laboratory - Cincinnati (EMSL-Cincinnati) conducts research to:

- Develop and evaluate methods to measure the presence and concentration of physical, chemical, and radiological pollutants in water, wastewater, bottom sediments, and solid waste.
- Investigate methods for the concentration, recovery, and identification of viruses, bacteria, and other microbiological organisms in water; and to determine the responses of aquatic organisms to water quality.
- Develop and operate an Agency-wide quality assurance program to assure standardization and quality control of systems for monitoring water and wastewater.
- Develop and operate a computerized system for instrument automation leading to improved data collection, analysis, and quality control.

The methods described in this report can be used to measure the bacteriological quality of recreational, shellfish growing, ambient, and potable waters. A direct relationship between the density of enterococci and *E. coli* in water and the occurrence of swimming-associated gastroenteritis has been established through epidemiological studies of marine and fresh water bathing beaches. These studies have led to the development of criteria which can be used to establish recreational water standards based on recognized health effects-water quality relationships.

Robert L. Booth

Robert L. Booth, Director
Environmental Monitoring and Support
Laboratory - Cincinnati

TABLE OF CONTENTS

FIGURES

ACKNOWLEDGEMENTS

This is to acknowledge the major contributions of Alfred Dufour and Theodore Ericksen of the Toxicology and Microbiology Division (TMD), Health Effects Research Laboratory (HERL) in preparation of the original methods, and their assistance and that of Robert Bordner, Biological Methods Branch, and John Winter and Paul Britton, Quality Assurance Branch, Environmental Monitoring and Support Laboratory - Cincinnati (EMSL-Cincinnati), U.S. Environmental Protection Agency (USEPA) in preparing the final protocol and in completing the formal method validation studies.

The participation of the following individuals in the interlaboratory testing of these methods is also gratefully acknowledged:

Rebecca Calderon
Dept. of Epidemiology and
Public Health
School of Medicine
Yale University
New Haven, CT

Wendy Davis-Hoover
TMD, HERL
USEPA
Cincinnati, OH

Rodger Fukjioka
Water Resources Research Center
University of Hawaii at Manoa
Honolulu, HA

John Haines
Biological Methods Branch
EMSL-Cincinnati
USEPA
Cincinnati OH

Linda Hopkins
Technion International
Cincinnati, OH

Don Johnstone
Environmental Engineering Department
Washington State University
Pullman, WA

Vincent Olivieri
Dept. of Environmental Health
Johns Hopkins University
Baltimore, MD

Scott Rippey
Construction Battalion Center
U.S. Public Health Service
Department of Health and
Human Services
North Kingston, RI

William Randolph
Bionetic Corporation
Cincinnati, OH

George J. Vasconcelos
Region 10 Laboratory
USEPA
Manchester, WA

Larry Vlassoff
Laboratory Branch
Ontario Ministry of Environment
Rexdale, Ontario, Canada

TEST METHOD

ESCHERICHIA COLI IN WATER BY THE MEMBRANE FILTER PROCEDURE

METHOD 1103.1
1985

1. Citation

2. Scope and Application

2.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of Escherichia coli. Because the bacterium is a natural inhabitant only of the intestinal tract of warm-blooded animals, its presence in water samples is an indication of fecal pollution and the possible presence of enteric pathogens.

2.2 The E. coli test is used as a measure of recreational water quality. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on established relationships between health effects and water quality. The significance of finding E. coli in recreational water samples is the direct relationship between the density of E. coli and the risk of gastrointestinal illness associated with swimming in the water (1).

2.3 The test for E. coli can be applied to fresh, estuarine and marine waters.

2.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of E. coli levels in water can be detected and enumerated.

3. Summary

3.1 The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (2). A water sample is filtered through the membrane which retains the bacteria. After filtration, the membrane containing the bacterial cells is placed on a selective and differential medium, mTEC, incubated at 35°C for 2 h to resuscitate injured or stressed bacteria, and then incubated at 44.5°C for 22 h. Following incubation, the filter is transferred to a filter pad saturated with urea substrate. After 15 min, yellow or yellow-brown colonies are counted with the aid of a fluorescent lamp and a magnifying lens.

4. Definition

4.1 In this method, E. coli are those bacteria which produce yellow or yellow-brown colonies on a filter pad saturated with urea substrate broth after primary culturing on mTEC medium.

5. Interferences

5.1 Water samples containing colloidal or suspended particulate material can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

6. Safety Precautions

6.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using, and disposing of cultures, reagents and materials and while operating sterilization equipment.

6.2 Mouth-pipetting is prohibited.

7. Apparatus and Equipment

7.1 Glass lens, 2-5X magnification, or stereoscopic microscope.

7.2 Lamp with cool, white fluorescent tube and diffuser.

7.3 Hand tally or electronic counting device.

7.4 Pipet container, stainless steel, aluminum, or borosilicate glass, for glass pipets.

7.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 Graduated cylinders, covered with aluminum foil or kraft paper and sterile.

7.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterile.

7.8 Ultraviolet unit for sterilizing the filter funnel between filtrations (optional).

7.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.

7.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 Flask for safety trap, placed between the filter flask and the vacuum source.

7.12 Forceps, straight or curved, with smooth tips to handle filters without damage.

7.13 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

7.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing inoculation loops.

7.15 Thermometer, checked against a National Bureau of Standards (NBS) certified thermometer, or one traceable to an NBS thermometer.

7.16 Petri dishes, sterile, plastic, 50 x 12 mm, with tight-fitting lids, or 60 x 15 mm, glass or plastic, with loose-fitting lids. 100 x 15 mm dishes may also be used.

7.17 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1-100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1-10 dilutions.

7.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

7.19 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 $\pm$ 0.02 µm pore size.

7.20 Absorbent pads, sterile, 47 mm diameter (usually supplied with membrane filters).

7.21 Inoculation loops, at least 3 mm diameter, and needles, nichrome and platinum wire, 26 B & S gauge, in suitable holders. Disposable applicator sticks or plastic loops are alternatives to inoculation loops. Note: A platinum loop is required for the cytochrome oxidase test in 15.3.

7.22 Incubator maintained at 35 $\pm$ 0.5°C, with approximately 90 percent humidity if loose-lidded petri dishes are used.

7.23 Waterbath incubator maintained at 44.5 $\pm$ 0.2°C.

7.24 Waterbath maintained at 44-46°C for tempering agar.

7.25 Test tubes, 150 x 20 mm, borosilicate glass or plastic.

7.26 Test tubes, 75 x 10 mm, borosilicate glass.

7.27 Test tube caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.28 Test tubes, screw-cap, 125 x 16 mm or other appropriate size.

7.29 Filter paper.

8. Reagents and Materials

8.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (3). The agar used in preparation of culture media must be of microbiological grade.

8.2 Whenever possible, use commercial culture media as a means of quality control.

8.3 Purity of Water: Reagent water conforming to Specification D1193, Type II water, ASTM Annual Book of Standards (4).

8.4 Buffered Dilution Water

8.4.1 Composition:

Sodium Dihydrogen Phosphate	0.58 g
Sodium Monohydrogen Phosphate	2.50 g
Sodium Chloride	8.50 g

8.4.2 Preparation: Dissolve the ingredients in 1 L of reagent water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb pressure) for 15 min. Final pH should be 7.4 ± 0.2

8.5. mTEC Agar (Difco 0334-15-0)

8.5.1 Composition:

Proteose Peptone	5.0 g
Yeast Extract	3.0 g
Lactose	10.0 g
NaCl	7.5 g
Dipotassium Phosphate	3.3 g
Monopotassium Phosphate	1.0 g
Sodium Lauryl Sulfate	0.2 g
Sodium Desoxycholate	0.1 g
Brom Cresol Purple	0.08 g
Brom Phenol Red	0.08 g
Agar	15.0 g

8.5.2 Preparation: Add 45.26 g of dehydrated mTEC medium to 1 L of reagent water in a flask and heat to boiling, until ingredients dissolve. Autoclave at 121°C (15 lb pressure) for 15 minutes and cool in a 44-46°C waterbath. Pour the medium into each 50 x 10 mm culture dish to a 4-5 mm depth (approximately 4-6 mL) and allow to solidify. Final pH should be 7.3 ± 0.2. Store in a refrigerator.

8.6 Urea Substrate Medium

8.6.1 Composition:

Urea	2.0 g
Phenol red	0.01 g

8.6.2 Preparation: Add dry ingredients to 100 mL reagent water in a flask. Stir to dissolve and adjust to pH 5.0 with a few drops of 1N HCl. The substrate solution should be a straw yellow color at pH 5.0.

8.7 Nutrient Agar (Difco 0001-02, BBL 11471)

8.7.1 Composition:

Peptone	5.0 g
Beef Extract	3.0 g
Agar	15.0 g

8.7.2 Preparation: Add 23 g of dehydrated nutrient agar to 1 L of reagent water and mix well. Heat in a boiling waterbath to dissolve the agar completely. Dispense in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. Remove tubes and slant. The final pH should be 6.8 $\pm$ 0.2.

8.8 Tryptic Soy Broth (Difco 0370-02 Trypticase Soy Broth (BBL 11767)

8.8.1 Composition:

Tryptone or Trypticase	17.0 g
Soytone or Phytone	3.0 g
Sodium Chloride	5.0 g
Dextrose	2.5 g
Dipotassium Phosphate	2.5 g

8.8.2 Preparation: Add 30 g of dehydrated tryptic soy broth to 1 L of reagent water. Warm the broth and mix gently to dissolve the medium completely. Dispense in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. The final pH should be 7.3 $\pm$ 0.2.

8.9 Simmons' Citrate Agar (BBL 11619, Difco 0091-02)

8.9.1 Composition:

Magnesium Sulfate	0.2 g
Monoammonium Phosphate	1.0 g
Dipotassium Phosphate	1.0 g
Sodium Citrate	2.0 g
Sodium Chloride	5.0 g
Brom Thymol Blue	0.08 g
Agar	15.0 g

8.9.2 Preparation: Add 24.28 g of Simmons' citrate agar to 1 L of reagent water. Heat in boiling waterbath with mixing for complete solution. Dispense in screw-cap tubes and sterilize at 121°C (15 lb pressure) for 15 min. Cool tubes and slant. The final pH should be 6.8 ± 0.2.

8.10 Tryptone 1% (Difco 0123-02) Tryptophane Broth (BBL 11920)

8.10.1 Composition:

Tryptone or Trypticase Peptone	10.0 g
Reagent Water	1 L

8.10.2 Preparation: Add 10 g of tryptone or trypticase peptone to 1 L of reagent water and heat with mixing until dissolved. Dispense in five mL volumes in tubes and autoclave at 121°C (15 lbs. pressure) for 15 min. The final pH should be 7.2 ± 0.2.

8.11 EC Broth (Difco 0314-02) EC Broth (BBL 11187)

8.11.1 Composition:

Tryptose or Trypticase Peptone	20.0 g
Lactose	5.0 g
Bile Salts No. 3 or Bile Salts Mixture	1.5 g
Dipotassium Phosphate	4.0 g
Monopotassium Phosphate	1.5 g
Sodium Chloride	5.0 g

Final pH: 6.9 ± 0.2

8.11.2 Preparation: Add 37 grams of dehydrated EC medium to 1 L of reagent water and warm to dissolve completely. Dispense into fermentation tubes (150 x 20 mm tubes containing inverted 75 x 10 mm vials). Sterilize at 121°C (15 lb pressure) for 15 min. The final pH should be 6.9 ± 0.2.

8.12 Cytochrome Oxidase Reagent: N, N, N', N' tetramethyl-p-phenylenediamine dihydrochloride, 1% aqueous solution.

8.13 Kovac's Indole Reagent: Dissolve 10 g p-dimethylaminobenzaldehyde in 150 mL amyl or isoamyl alcohol and then slowly add 50 mL concentrated hydrochloric acid and mix.

9. Sample Collection, Preservation and Holding Times

9.1 Sampling procedures are described in detail in the USEPA microbiology methods manual, Section II, A (5). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these rules should not be analyzed.

9.1.1 Storage Temperature and Handling Conditions

Ice or refrigerate water samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

9.1.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 h between collection and initiation of analyses.

10. Calibration and Standardization

10.1 Check temperatures in incubators daily to insure operation within stated limits.

10.2 Check thermometers at least annually against an NBS certified thermometer or one traceable to NBS. Check mercury columns for breaks.

11. Quality Control

11.1 See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, C (5).

12. Procedure

12.1 Prepare the mTEC agar and urea substrate as directed in 8.5 and 8.6.

12.2 Mark the petri dishes and report forms with sample identification and sample volumes.

12.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

12.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly and measure the desired volume of sample or dilution into the funnel.

12.5 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge of pollution level, to produce 20-80 E. coli colonies on the membranes. Sample volumes of 1-100 mL are normally tested at half-log intervals, for example 100, 30, 10, 3 mL, etc.

12.6 Smaller sample size or sample dilutions can be used to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or sample dilution may be filtered and the results combined.

12.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

12.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the mTEC agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 35°C for 2 h.

12.9 After two hours incubation at 35°C, transfer the plates to Whirl-Pak bags, seal, and place inverted in a 44.5°C waterbath for 22-24 h.

12.10 After 22-24 h, remove the dishes from the waterbath. Place absorbent pads in new petri dishes or the lids of the same petri dishes, and saturate with urea broth. Aseptically transfer the membranes to absorbent pads saturated with urea substrate and hold at room temperature.

12.11 After 15-20 min incubation on the urea substrate at room temperature, count and record the number of yellow or yellow-brown colonies on those membrane filters ideally containing 20-80 colonies.

13. Calculation of Results

13.1 Select the membrane filter with the number of colonies within the acceptable range (20-80) and calculate the count per 100 mL according to the general formula:

$$\text{E. coli/100 mL} = \frac{\text{No. E. coli colonies counted}}{\text{Volume in mL of sample filtered}} \times 100 \text{ mL}$$

13.2 See general counting rules in the USEPA microbiology methods manual, Part II, C, 3.5 (5).

14. Reporting Results

14.1 Report the results as E. coli per 100 mL of sample.

15. Verification Procedure

15.1 Yellow or yellow-brown colonies from the urease test can be verified as E. coli. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure with initial use of the test and with changes in sample sites, lots of commercial media or major ingredients in media compounded in the laboratory. The verification procedure follows:

15.1.1 Using a sterile inoculation loop, transfer growth from the centers of at least 10 well-isolated typical colonies to nutrient agar plates or slants and to trypticase soy broth. Incubate the agar and broth cultures for 24 h at 35°C.

15.1.2 After incubation remove a generous portion of material from the nutrient agar with a platinum loop and deposit on the surface of filter paper that has been saturated with cytochrome oxidase reagent prepared fresh that day. A positive test is indicated within 15 s by the development of a deep purple color where the bacteria were deposited.

15.1.3 Transfer growth from the trypticase soy broth to Simmons' citrate agar, tryptone broth, and EC broth in a fermentation tube. Incubate the Simmons' citrate agar for 24 h and tryptone broth for 48 h at 35°C. Incubate the EC broth at 44.5°C in a waterbath for 24 h. The water level must be above the level of the EC broth in the tube. Add one-half mL of Kovac's indole reagent to the 48-h tryptone broth culture and shake the tube gently. A positive test for indole is indicated by a deep red color which develops in the alcohol layer. E. coli is EC gas positive, indole positive, oxidase negative, and does not grow on citrate medium.

15.1.4 Alternatively, commercially available multi-test identi-identification systems may be used to verify colonies. Inoculate the colonies into an identification system for Enterobacteriaceae that includes lactose fermentation and/or O-nitrophenyl- -D-galactopyranoside (ONPG) and cytochrome oxidase test reactions.

16. Precision and Bias

16.1 Performance Characteristics

16.1.1 Precision - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard

deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. The mTEC method precision was found to be fairly representative of what would be expected from counts with a Poisson distribution (2).

16.1.2 Bias - The persistent positive or negative deviation of the average value of the method from the assumed or accepted true value. The bias of the mTEC method has been reported to be -2% of the true value (2).

16.1.3 Specificity - The ability of a method to select and or distinguish the target bacteria under test from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The false positive rate reported for mTEC medium averaged 9% for marine and fresh water samples. Less than 1% of the E. coli colonies observed gave a false negative reaction (2).

16.1.4 Upper Counting Limit (UCL) - That colony count above which there is an unacceptable counting error. The error may be due to overcrowding or antibiosis. The UCL for E. coli on mTEC medium has been reported as 80 colonies per filter (2).

16.2 Collaborative Study Data

16.2.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate. The data were reported to the Environmental Monitoring and Support Laboratory - Cincinnati, U.S. Environmental Protection Agency, for statistical calculations.

16.2.2 The results of the study are shown in Figure 1 where S_O equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not show any difference among the water types analyzed.

16.2.3 By linear regression, the precision of the method can be generalized as:

$$S_O = 0.028 \text{ count/100 mL} + 6.11 \text{ (dilution factor) and}$$

$$S_B = 0.233 \text{ count/100 mL} + 0.82 \text{ (dilution factor)}$$

where dilution factor $= \dfrac{100}{\text{volume of original sample filtered}}$

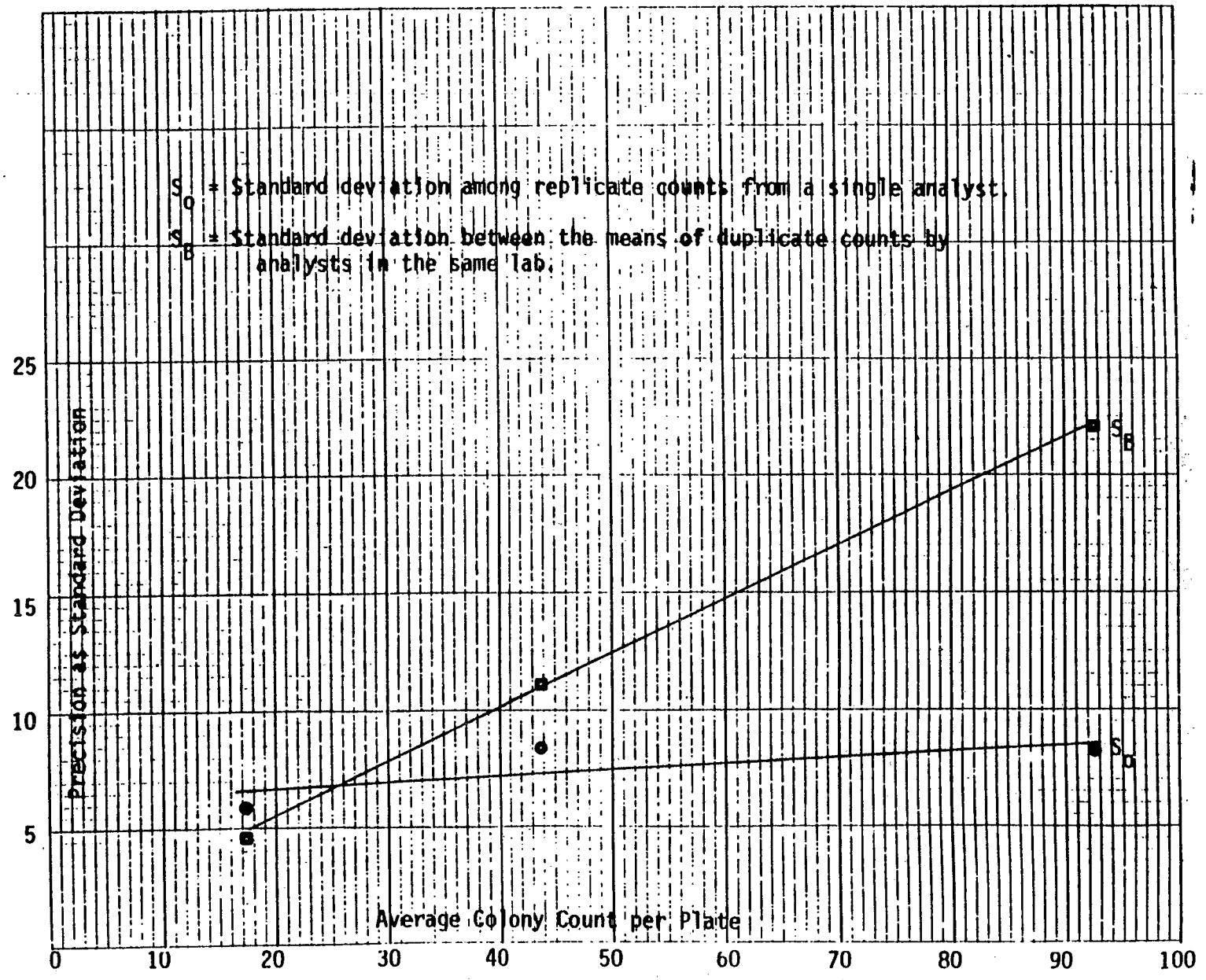

FIGURE 1. Precision Estimates for E. coli in Water by the Membrane Filter/mTEC Procedure.

16.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of E. coli. The mean count (x) and the overall standard deviation of the counts (S_T) (which includes the variability among laboratories for this standardized E. coli sample) were 31.6 colonies/membrane and 7.61 colonies/membrane, respectively.

References

1. Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," Amer. Jour. Public Health 69, 690-696, 1979.

2. Dufour, A., E. Strickland, and V. Cabelli., "Membrane Filter Method for Enumerating Escherichia coli," Appl. and Environ. Microbiol. 41, 1152-1158, 1981.

3. "Reagent Chemicals," American Chemical Society Specifications, 6th Edition, Am. Chem. Soc., Washington, D.C., 1981. For suggestions on the testing of reagents not listed by the American Chemical Society, see Reagent Chemicals and Standards, Joseph Rosin, D. Van Nostrand Co., Inc., Princeton, N.J., 1967, and the United States Pharmacopeia, Nineteenth Edition, United States Pharmacopeial Convention, Inc., Rockville, Md., 1974.

4. Annual Book of ASTM Standards, Vol. 11.01, Water, American Society for Testing and Materials, Philadelphia, PA., 1985.

5. Bordner, R., J. A. Winter and P. V. Scarpino (eds.), Microbiological Methods for Monitoring the Environment. Water and Wastes, EPA-600/8-78-077, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory - Cincinnati, Cincinnati, Ohio, 1978.

TEST METHOD

ENTEROCOCCI IN WATER BY THE MEMBRANE FILTER PROCEDURE

METHOD 1106.1
1985

1. Citation

2. Scope and Application

2.1 This method describes a membrane filter (MF) procedure for the detection and enumeration of the enterococci bacteria in water. The enterococci are commonly found in the feces of humans and other warm-blooded animals. Although some strains are ubiquitous and not related to fecal pollution, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens.

2.2 The enterococci test measures the bacteriological quality of recreational waters. Epidemiological studies have led to the development of criteria which can be used to promulgate recreational water standards based on the established relationship between health effects and water quality. The signficance of finding enterococci in recreational water samples is the direct relationship between the density of enterococci in the water and swimming-associated gastroenteritis studies of marine and fresh water bathing beaches (1).

2.3 The test for enterococci can be applied to potable, fresh, estuarine, marine, and shellfish growing waters.

2.4 Since a wide range of sample volumes or dilutions can be analyzed by the MF technique, a wide range of enterococci levels in water can be detected and enumerated.

3. Summary

3.1 The MF method provides a direct count of bacteria in water based on the development of colonies on the surface of the membrane filter (2). A water sample is filtered through the membrane which retains the bacteria. Following filtration, the membrane containing the bacterial cells is placed on a selective medium, mE agar, and incubated for 48 h at 41°C. After incubation, the filter is transferred to EIA agar and held at 41°C for 20 min. Pink to red enterococci colonies will develop a black or reddish-brown precipitate on the underside of the filter. These colonies are counted with a fluorescent lamp and a magnifying lens.

4. Definition

4.1 In this method, enterococci are those bacteria which produce pink to red colonies with black or reddish-brown precipitate after incubation

on mE agar and subsequent transfer to EIA medium. Enterococci include Streptococcus faecalis, Streptococcus faecium, Streptococcus avium, and their variants.

5. Interferences

5.1 Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration, or cause spreading of bacterial colonies which could interfere with identification of target colonies.

6. Safety Precautions

6.1 The analyst/technician must know and observe the normal safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents, and materials, and while operating sterilization equipment.

6.2 Mouth-pipetting is prohibited.

7. Apparatus and Equipment

7.1 Glass lens with magnification of 2-5X or stereoscopic microscope.

7.2 Lamp, with a cool, white fluorescent tube.

7.3 Hand tally or electronic counting device.

7.4 Pipet container, stainless steel, aluminum or borosilicate glass, for glass pipets.

7.5 Pipets, sterile, T.D. bacteriological or Mohr, glass or plastic, of appropriate volume.

7.6 Graduated cylinders, 100-1000 mL, covered with aluminum foil or kraft paper and sterile.

7.7 Membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper and sterile.

7.8 Ultraviolet unit for sterilizing the filter funnel between filtrations (optional).

7.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air, can be used.

7.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing. A filter manifold to hold a number of filter bases is optional.

7.11 Flask for safety trap placed between the filter flask and the vacuum source.

7.12 Forceps, straight or curved, with smooth tips to handle filters without damage.

7.13 Ethanol, methanol or isopropanol in a small, wide-mouth container, for flame-sterilizing forceps.

7.14 Burner, Bunsen or Fisher type, or electric incinerator unit for sterilizing loops and needles.

7.15 Thermometer, checked against a National Bureau of Standards (NBS) certified thermometer, or one traceable to an NBS thermometer.

7.16 Petri dishes, sterile, plastic, 50 x 12 mm, with tight-fitting lids.

7.17 Bottles, milk dilution, borosilicate glass, screw-cap with neoprene liners, marked at 99 mL for 1-100 dilutions. Dilution bottles marked at 90 mL or tubes marked at 9 mL may be used for 1-10 dilutions.

7.18 Flasks, borosilicate glass, screw-cap, 250-2000 mL volume.

7.19 Membrane filters, sterile, white, grid marked, 47 mm diameter, with 0.45 $\pm$ 0.02 µm pore size.

7.20 Inoculation loops, at least 3-mm diameter, and needles, nichrome or platinum wire, 26 B & S gauge, in suitable holders.

7.21 Incubator maintained at 41 $\pm$ 0.5°C.

7.22 Waterbath maintained at 44-46°C for tempering agar.

7.23 Test tubes, 150 x 20 mm, borosilicate glass or plastic.

7.24 Caps, aluminum or autoclavable plastic, for 20 mm diameter test tubes.

7.25 Test tubes, screw-cap, borosilicate glass, 125 x 16 mm or other appropriate size.

8. Reagents and Materials

8.1 Purity of Reagents: Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society (3). The agar used in preparation of culture media must be of microbiological grade.

8.2 Whenever possible, use commercial culture media as a means of quality control.

8.3 Purity of Water: Reagent water conforming to Specification D1193, Type II, Annual Book of ASTM Standards (4).

8.4 Buffered Dilution Water

8.4.1 Composition:

Sodium Dihydrogen Phosphate	0.58 g
Sodium Monohydrogen Phosphate	2.50 g
Sodium Chloride	8.50 g

8.4.2 Preparation: Dissolve the ingredients in 1 L of reagent water in a flask and dispense in appropriate amounts for dilutions in screw-cap bottles or culture tubes and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb pressure) for 15 min. The final pH should be 7.4 ± 0.2.

8.5 mE Agar (Difco 0333-15-1)

8.5.1 Composition of Basal Medium:

Peptone	10.0 g
Sodium Chloride	15.0 g
Yeast Extract	30.0 g
Esculin	1.0 g
Actidione	0.05 g
Sodium Azide	0.15 g
Agar	15.0 g

8.5.2 Preparation of basal medium: Add 71.2 g of dehydrated mE basal medium to 1 L of reagent grade water in a flask and heat to boiling until ingredients dissolve. Autoclave at 121°C and 15 lb pressure for 15 min and cool in a 44-46°C water bath.

8.5.3 Reagents added after sterilization: Mix 0.25 g nalidixic acid in 5 mL reagent grade water, add 0.2 mL of 10 N NaOH to dissolve, and add to the basal medium. Add 0.15 g triphenyl tetrazolium chloride separately to the basal medium and mix.

8.5.4 Preparation of mE Agar: Pour the mE agar into 50 mm petri dishes to a 4-5 mm depth (approximately 4-6 mL), and allow to solidify. The final pH of medium should be 7.1 ± 0.2. Store in a refrigerator.

8.6 EIA Substrate Agar (Difco 0488-15-4)

8.6.1 Composition:

Esculin	1.0 g
Ferric Citrate	0.5 g
Agar	15.0 g

Preparation: Add 16.5 g of dehydrated EIA medium to 1 L of reagent grade water in a flask and heat to boiling until ingredients are dissolved. Autoclave the EIA medium at 121°C and 15 lb pressure for 15 min and cool in a 44-46°C water bath. After cooling, pour the medium into 50-mm petri dishes to a depth of 4-5 mm (approximately 4-6 mL) and allow to solidify. The final pH should be 7.1 ± 0.2 before autoclaving. Store in a refrigerator.

8.7 Brain Heart Infusion (BHI) (Difco 0037-02, BBL 11058)

8.7.1 Composition:

Calf Brain Infusion	200.0 g
Beef Heart Infusion	250.0 g
Peptone	10.0 g
Sodium Chloride	5.0 g
Disodium Phosphate	2.5 g
Dextrose	2.0 g

8.7.2 Preparation: Dissolve 37 g of dehydrated brain heart infusion in 1 L of reagent grade water. Dispense in 8-10 mL volumes in screw-cap tubes and autoclave at 121°C (15 lb pressure) for 15 min. If the medium is not used the same day as prepared and sterilized, heat in boiling water bath for several min to remove absorbed oxygen, and cool quickly without agitation, just prior to inoculation. The final pH should be 7.4 ± 0.2.

8.8 Brain Heart Infusion (BHI) Broth with 6.5% NaCl

8.8.1 Composition: Brain heart infusion broth with 6.5% NaCl is the same as BHI broth in 8.7 with additional NaCl.

8.8.2 Preparation: Add 60.0 g NaCl per liter of medium. Since most commercially available dehydrated media contain sodium chloride, this amount is taken into consideration in determining the final NaCl percentage above.

8.9 Brain Heart Infusion Agar (Difco 0418-02, BBL 11064)

8.9.1 Composition: Brain heart infusion agar contains the same components as BHI (see 8.7) with the addition of 15.0 g of agar per L of BHI broth.

8.9.2 Preparation: Heat to boiling until ingredients are dissolved. Dispense 10-12 mL of medium in screw-cap test tubes and sterilize for 15 min at 121°C (15 lb pressure). Slant after sterilization. The final pH should be 7.4 ± 0.2.

8.10 Bile Esculin Agar (BEA) (Difco 0879)

8.10.1 Composition:

Bacto Beef Extract	3.0 g
Bacto Peptone	5.0 g
Bacto Oxgall	40.0 g
Bacto Esculin	1.0 g
Ferric Citrate	0.5 g
Bacto Agar	15.0 g

8.10.2 Preparation: Add 64.5 g of dehydrated BEA to 1 L reagent water and heat to boiling to dissolve completely. Dispense in 8-10 mL volumes in tubes for slants or into flasks for subsequent plating. Autoclave at 121°C at 15 lb pressure for 15 min. Overheating may cause darkening of the medium. Cool to 44-46°C and dispense into sterile petri dishes. The final pH should be 6.6 ± 0.2. Store in a refrigerator.

9. Sample Collection, Preservation and Holding Times

9.1 Sampling procedures are described in detail in the USEPA microbiology methods manual, Section II, A (5). Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples should not be analyzed if these conditions are not met.

9.1.1 Storage Temperature and Handling Conditions

Ice or refrigerate bacteriological samples at a temperature of 1-4°C during transit to the laboratory. Use insulated containers to assure proper maintenance of storage temperature. Take care that sample bottles are not totally immersed in water during transit or storage.

9.1.2 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 h between collection and initiation of analyses.

10. Calibration and Standardization

10.1 Check temperatures in incubators daily to insure operation within stated limits.

10.2 Check thermometers at least annually against an NBS certified thermometer or one traceable to NBS. Check mercury columns for breaks.

11. Quality Control

11.1 See recommendations on quality control for microbiological analyses in the USEPA microbiology methods manual, Part IV, C (5).

12. Procedure

12.1 Prepare the mE agar as directed in 8.5.

12.2 Mark the petri dishes and report forms with sample identification and sample volumes.

12.3 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base; the membrane filter is now held between the funnel and the base.

12.4 Shake the sample bottle vigorously about 25 times to distribute the bacteria uniformly, and measure the desired volume of sample or dilution into the funnel.

12.5 For ambient surface waters and wastewaters, select sample volumes based on previous knowledge of pollution level, to produce 20-60 enterococci colonies on membranes. Sample volumes of 1-100 mL are normally tested at half log intervals, for example 100, 30, 10, 3mL, etc.

12.6 Smaller sample size or sample dilution can be used to minimize the interference of turbidity or high bacterial densities. Multiple volumes of the same sample or dilution of sample may be filtered and the results combined.

12.7 Filter the sample and rinse the sides of the funnel at least twice with 20-30 mL of sterile buffered rinse water. Turn off the vacuum and remove the funnel from the filter base.

12.8 Use sterile forceps to aseptically remove the membrane filter from the filter base and roll it onto the ME agar to avoid the formation of bubbles between the membrane and the agar surface. Reseat the membrane if bubbles occur. Close the dish, invert, and incubate at 41 $\pm$ 0.5°C for 48 h.

12.9 After incubation, transfer the membranes to EIA agar plates which have been at room temperature for 20-30 min, and incubate at 41°C for 20 min.

12.10 After incubation, count and record colonies on those membrane filters containing, if practical, 20-60 pink-to-red colonies with

black or reddish-brown precipitate on the underside of the membrane. Use magnification for counting and a small fluorescent lamp to give maximum visibility of colonies.

13. Calculation of Results

Use the following general rules to calculate the enterococci count per 100 mL of sample:

13.1 Select and count membranes with ideally 20-60 pink to red colonies with black or reddish-brown precipitate on the underside. Calculate the final value using the formula:

$$\text{Enterococci/100 mL} = \frac{\text{No. of enterococci colonies}}{\text{Volume of sample filtered (mL)}} \times 100$$

13.2 See the USEPA microbiology manual, Part II, Section C, 3.5, for general counting rules.[5]

14. Reporting Results

14.1 Report the results as enterococci per 100 mL of sample.

15. Verification Procedure

15.1 Pink to red colonies with black or reddish-brown precipitate after incubation on EIA agar can be verified as enterococci. Verification of colonies may be required in evidence gathering, and is also recommended as a QC procedure upon initial use of the test and with changes in sample sites, lots of commercial media, or major ingredients in media compounded in the laboratory. The verification procedure follows:

15.2 Using a sterile inoculating needle, transfer cells from the centers of at least 10 well-isolated typical colonies into a brain heart infusion broth (BHI) tube and onto a BHI slant. Incubate broth tubes for 24 h and slants for 48 h at 35 $\pm$ 0.5°C.

15.3 After 24 h incubation, transfer a loopful of material from each BHI broth tube to:

a. Bile Esculin Agar (BEA) and incubate at 35 $\pm$ 0.5°C for 48 h.

b. BHI Broth and incubate at 45 $\pm$ 0.5°C for 48 h.

c. BHI Broth with 6.5% NaCl and incubate at 35 $\pm$ 0.5°C for 48 h.

15.4 Observe for growth.

15.5 After 48 h incubation, apply a gram stain to growth from each BHI agar slant.

15.6 Gram positive cocci which grow in BEA, BHI Broth at 45°C, and BHI Broth + 6.5% NaCl, and hydrolyze esculin, are verified as enterococci.

16. Precision and Bias

16.1 Performance Characteristics

16.1.1 Precision - The degree of agreement of repeated measurements of the same parameter expressed quantitatively as the standard deviation or as the 95% confidence limits of the mean computed from the results of a series of controlled determinations. Precision of the mE method was established by Levin et al. (2) who indicated that the method did not exceed the expected limits for counts having the Poisson distribution.

16.1.2 Bias - The persistent positive or negative deviation of the results from the assumed or accepted true value. The bias of the enterococci MF method with mE Agar has been reported to be +2% of the true value (2).

16.1.3 Specificity - The ability of a method to select and/or distinguish the target bacteria from other bacteria in the same water sample. The specificity characteristic of a method is usually reported as the percent of false positive and false negative results. The specificity for this medium as reported for various environmental water samples was 10% false positive and 11.7% false negative (2).

16.2 Collaborative Study Data

16.2.1 A collaborative study was conducted among eleven volunteer laboratories, each with two analysts who independently tested local fresh and marine recreational waters and sewage treatment plant effluent samples, in duplicate. The data were reported to the Environmental Monitoring and Support Laboratory - Cincinnati, U.S. Environmental Protection Agency, for statistical analyses.

16.2.2 The results of the study are shown in Figure 1 where S_O equals the pooled standard deviation among replicate counts from a single analyst for three groupings (counts less than 30, counts from 30 to 50, and counts greater than 50) and S_B equals the pooled standard deviation between means of duplicates from analysts in the same laboratory for the same groupings. The precision estimates from this study did not differ with the water types tested.

16.2.3 By linear regression, the precision of the method can be generalized as:

$$S_O = 0.103 \text{ count/100 mL} + 2.42 \text{ (dilution factor) and}$$

$$S_B = 0.152 \text{ count/100 mL} + 5.16 \text{ (dilution factor)}$$

where dilution factor = $\frac{100}{\text{volume of original sample filtered}}$

16.2.4 Because of the instability of microbial populations in water samples, each laboratory analyzed its own sample series and no full measure of recovery or bias was possible. However, all laboratories analyzed a single surrogate sample prepared from a freeze-dried culture of Streptococcus faecalis. The mean count ($\bar{x}$) and the standard deviation of the counts (S_T) (including the variability among laboratories for this standardized enterococci sample) were 32.5 colonies/ membrane and 9.42 colonies/membrane, respectively.

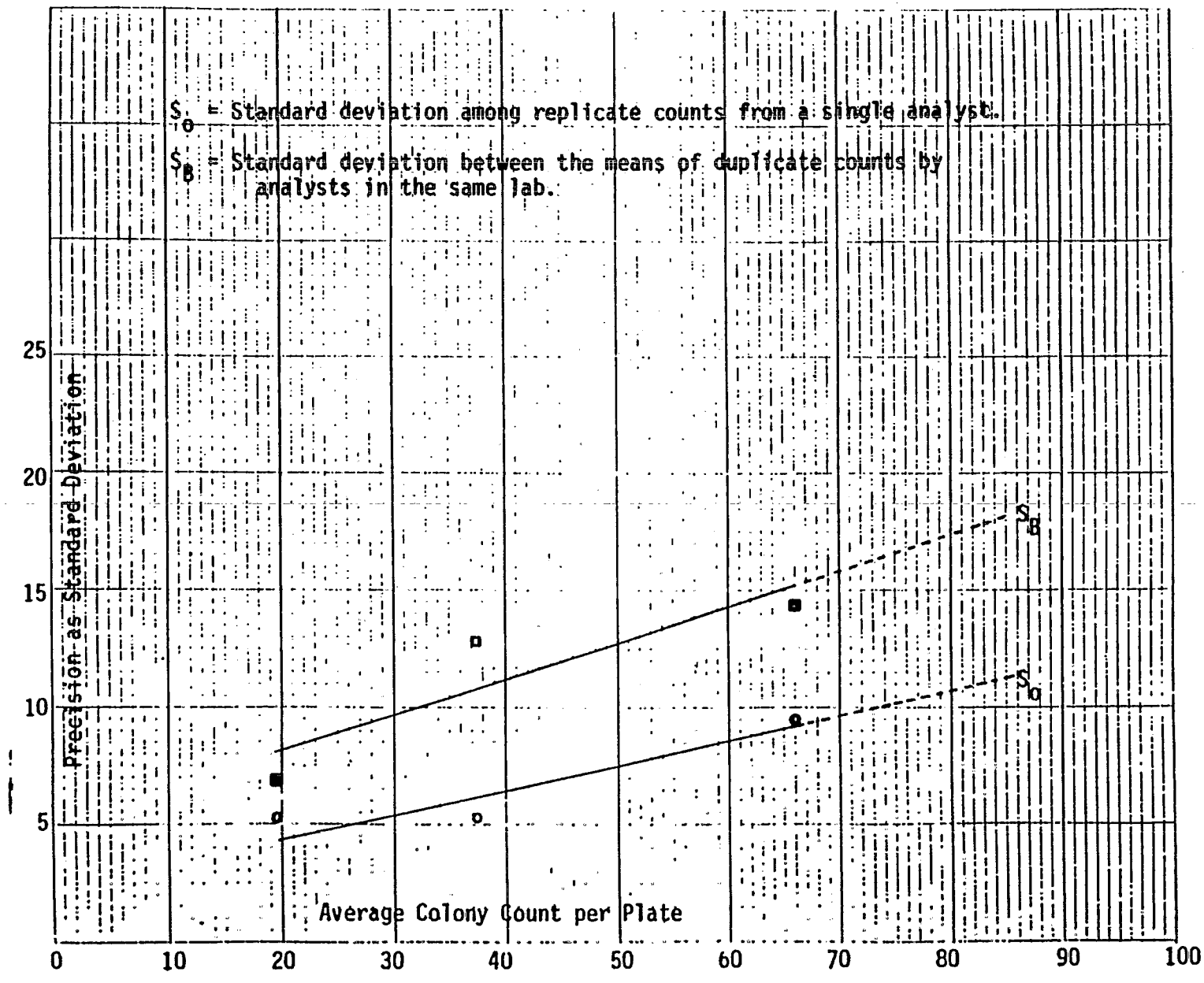

FIGURE 1. Precision Estimates for Enterococci in Water by the Membrane Filter/mE Procedure

References

1. Cabelli, V. J., A. P. Dufour, M. A. Levin, L. J. McCabe, and P. W. Haberman, "Relationship of Microbial Indicators to Health Effects at Marine Bathing Beaches," Amer. Jour. Public Health, 69, 690-696, 1979.

2. Levin, M. A., J. R. Fischer and V. J. Cabelli, "Membrane Filter Technique for Enumeration of Enterococci in Marine Waters," Appl. Microbiol. 30, 66-71, 1975.

3. "Reagent Chemicals," American Chemical Society Specifications 6th Edition, Am. Chem. Soc., Washington, D.C., 1981. For suggestions of the testing of reagents not listed by the American Chemical Society, see Reagent Chemicals and Standards, Joseph Rosin, D. Van Nostrand, Co., Inc., Princeton, N.J., 1967, and the United States Pharmacopeia, Nineteenth Edition, United States Pharmacopeial Convention, Inc., Rockville, MD, 1974.

4. Annual Book of ASTM Standards, Vol. 11.01, Water, American Society for Testing and Materials, Philadelphia, PA., 1985.

5. Bordner, R., J. A. Winter and P. V. Scarpino (eds.), "Microbiological Methods for Monitoring the Environment, Water and Wastes," EPA-600/8-78-017, U.S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory - Cincinnati, Cincinnati, Ohio, 1978.